Improving bridge effect to overcome interspecific hybrid sterility by pyramiding hybrid sterile loci from Oryza glaberrima

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Article

Keywords: bridge effect, hybrid sterility, interspecific hybrid, near isogenic lines, Oryza sativa, Oryza. glaberrima

Posted Date: September 5th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3232995/v1

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Scientific Reports on December 27th, 2023. See the published version at https://doi.org/10.1038/s41598-023-49914-4.

Abstract

In order to evaluate the hybrid sterile effect and the bridging effect caused by the hybrid sterile locus, total nine NILs carrying one, two and three alleles of *O. glaberrima* were developed and used as test lines to cross with *O. sativa* and *O. glaberrima* accessions. The results showed that the degree of pollen sterility of F_1 hybrids between the NILs and Dianjingyou 1 has deepened with the increase of the number of alleles from *O. glaberrima* at the hybrid sterile loci. Under the action of three hybrid sterile loci, F_1 hybrids between the two cultivated rice species are almost completely sterile. On the other hand, the NILs carrying a single allele of *O. glaberrima* had limited effect on improving pollen fertility of interspecific hybrid. The pyramided lines containing multiple sterile alleles of *O. glaberrima* showed increased pollen fertility when crossing with *O. glaberrima* accessions. Further the backcrossing can be used to improve pollen and spikelet fertility of interspecific hybrids after the first cross. The report has potential for understanding the nature of interspecific hybrid sterility, and overcoming the interspecific hybrid F_1 pollen grain sterility between *O. sativa* and *O. glaberrima*.

Introduction

Asian cultivated rice (*Oryza sativa* L.) is a prime food crop world-wide. However, advance in genetic improvement of rice has encountered problems owing to the narrow genetic diversity and the bottleneck of further yield increase[1]. African cultivated rice (*O. glaberrima* Steud.) is deemed to be a potential source of useful genes for improving Asian cultivated rice by hybridization as both cultivated species have the same AA genome and similar sequence arrangement[2, 3]. However, there are strong reproductive barriers in the interspecific hybrids between the two cultivated species[4]. The F_1 hybrids barely produce any fertile pollen grains and as a result the valuable genes disappear along with hybrid sterility. Therefore, hybrid sterility is one of the main hindrances against the utilization of useful genes from the African cultivated rice into Asian cultivated rice.

To date, at least 10 hybrid sterile loci were reported as gamete eliminators or pollen killers between *O. sativa* and *O. glaberrima*[5], and two of them were cloned[6, 7, 8]. The cumulative effects of these sterile loci lead to complete male sterility in F_1 hybrids. *O. glaberrima* and *O. sativa* varieties possessed the genotype *S-glab* and *S-sati*, respectively, at the sterile *S* locus. Homozygotes of *S-sati/S-sati* and *S-glab/S-glab* show normal fertility, while *S-sati* gametes are aborted when the sporophytic plants have the heterozygous genotype *S-sati/S-glab* in an *O. sativa* background. It was reported that the *O. sativa* lines carrying the *S1-glab* allele from *O. glaberrima* can be used as bridge parents to improve the fertility of hybrids between *O. glaberrima* and *O. sativa*[9]. However, the bridge effect of others sterile loci and their pyramided lines remain unknown.

In our previous study, six hybrid sterile loci, *S1, S19, S20, S37*(t), *S38*(t), *S39*(t) were identified from the crosses between *O. glaberrima* and *O. sativa*[10, 11, 12]. The *S1* and *S37*(t) loci function as the “gamete eliminator”: both male and female gametes carrying the allele of *O. sativa* are aborted in the heterozygotes. The *S19*, *S20, S38(t), S39(t)* locus functions as the “pollen killer”: only male gametes carrying the allele of *O. sativa* are aborted in the heterozygotes.

In order to improve the bridge effect to overcome interspecific reproductive barrier, the near isogenic lines (NILs) carrying the single and multiple alleles from O. *glaberrima* on the hybrid sterility loci *S1, S19, S20, S37*(t), *S38*(t) and *S39*(t) were developed. The hybrid sterile effect and bridge effect of the hybrid sterility loci were investigated in this study.

Results

NIls with single and multiple hybrid sterile alleles from O. glaberrima were developed.

A set of NILs, designated as NIL*S1*, NIL*S19*, NIL*S20*, NIL*S37*(t), NIL*S38*(t), NIL*S39*(t), with the *O. glaberrima* allele at the locus for hybrid sterility *S1*, *S19*, *S20*, *S37*(t), *S38*(t) and *S39*(t) in the genetic background of DJY1 were developed by successive backcross and molecular marker selection using DJY1 as the recurrent parent after hybridization with five *O. glaberrima* assessions[10, 11, 12]. These NILs were detected by a survey of the genome with the whole-genome SNP array. All of them carried the target chromosomal segment from the *O. glaberrima* accession and the genetic background was similar to the recurrent parent DJY1 (Fig. 1).

Three NILs, NIL*S1*, NIL*S20* and NIL*S37*(t), carrying the allele of *O. glaberrima* on the hybrid sterile loci *S1, S20, S37*(t) were used to develop the pyramided sterile loci lines with molecular marker-assistant method and phenotype selection. The pollen and spikelet fertility for all the F_1 plants were evaluated and the SSR molecular markers linked with *S1*, *S20*, and *S37*(t) were used to confirm the true hybrid. Those multiple *S* allele lines were self-pollinated and the marker-assistant method was used to obtain homozygous *S* alleles lines. Experiments to create the targeted locus pyramid started with two crosses NIL*S1*×NIL*S20* and NIL*S1*×NIL*S37*(t). Subsequently, the F_1 plants from the cross NIL*S1*×NIL*S37*(t) were used as the female to cross with the NIL*S20* for the *S1, S20, S37*(t) pyramiding.

The F_2 self-pollinated plants from the cross of NIL*S1*×NIL*S37*(t) showing the normal pollen and spikelet fertility, and homozygous genotype of *O. glaberrima* on SSR markers linked with *S1* and *S37*(t) were selected as the pyramid line for *S1* and *S37*(t) loci. We designated the pyramided line as the NIL*S1S37*(t). In fact, all the F_2 self-pollinated plants showing the homozygous genotype of *O. glaberrima* on *S1* and *S37*(t) loci because the *S1* and *S37*(t) loci function as the “gamete eliminator” and as the result only the gametes carrying the allele of *O. glaberrima* survived (Table 1).

With the assistance of SSR markers linked with the *S1* and *S20* loci, the F_2 self-pollinated plants from the cross of NIL*S1*×NIL*S20* showing the homozygous genotype of *O. glaberrima* on the *S1* and *S20* loci were selected and the pyramid lines for the *S1* and *S20* alleles from *O. glaberrima* in *O. sativa* genetic background were obtained. There were two genotypes in the F_2 self-pollinated offspring from the cross of NIL*S1*×NIL*S20*: the one is homozygotes of *O. glaberrima* at *S1* and *S20* loci; and another is homozygote of *O.glaberrima* at *S1* locus while heterozygous at *S20* locus. Only the male gamete of *S1-glab* and *S20-glab* survived while the female gamete of *S1-glab, S20-glab* and *S20-sati* survived in the F_1 plants because *S1* locus functions as the “gamete eliminator”

and *S20* locus functions as the "pollen killer". The selected plants with homozygous genotypes of *O. glaberrima* on the *S1* and *S20* loci also showed the normal pollen and spilelet fertility (Table 1). We designated the pyramided line as the NIL*S1S20.*

For the pyramided line of alleles from *O. glaberrima* on the *S1*, *S20* and *S37*(t) loci in *O. sativa* genetic background, the SSR markers linked with *S1*, *S20* and *S37*(t) alleles were used to genotype the F_2 plants in the cross of of (NIL*S1*×NIL*S37*(t))×NIL*S20*. The plants with homozygous genotype of *O. glaberrima* on the all three loci and showing the normal pollen and spikelet fertility were obtained as the pyramided line with the three alleles from *O. glaberrima* (Table 1). We designated the pyramided line as the NIL*S1S20S37*(t).

Totally, nine NILs carrying one, two and three allele of *O. glaberrima* were used as test lines to cross with *O. sativa* and *O. glaberrima* accessions. The hybrid sterile effect and the bridging effect caused by the hybrid sterility locus were studied.

Table 1
Pollen grain, spikelet fertility and genotype of F1 plants in the crosses between DJY1 and the NILs

Material name	Generation	Pollen fertility (%)	Spikelet fertility (%)	*S1* Chr.6[a]		*S19* Chr.3		*S20* Chr.7		*S37*(t) Chr.1		*S3* Chr.
				RM190	RM587	RM3372	RM22	RM20847	RM20852	RM449	RM513	RM
DJY1	P	99.05 ± 0.25	92.10 ± 3.07	s[b]	s	s	s	s	s	s	s	s
NIL*S1*	P	99.25 ± 0.31	91.19 ± 4.62	g	g	s	s	s	s	s	s	s
NIL*S19*	P	98.43 ± 1.46	86.42 ± 8.95	s	s	g	g	s	s	s	s	s
NIL*S20*	P	99.31 ± 0.29	93.12 ± 3.89	s	s	s	s	g	g	s	s	s
NIL*S37*(t)	P	98.84 ± 0.57	92.13 ± 3.77	s	s	s	s	s	s	g	g	s
NIL*S38*(t)	P	99.00 ± 0.42	90.11 ± 1.53	s	s	s	s	s	s	s	s	g
NIL*S39*(t)	P	97.63 ± 1.08	93.30 ± 5.64	s	s	s	s	s	s	s	s	s
NIL*S1S20*	P	99.06 ± 0.38	77.08 ± 5.00	g	g	s	s	g	g	s	s	s
NIL*S1S37*(t)	P	98.78 ± 0.54	78.75 ± 3.87	g	g	s	s	s	s	g	g	s
NIL*S1S20S37*(t)	P	98.93 ± 0.39	79.37 ± 4.43	g	g	s	s	g	g	g	g	s
DJY1×NIL*S1*	F_1	47.83 ± 1.85	51.97 ± 2.12	H	H	s	s	s	s	s	s	s
DJY1×NIL*S19*	F_1	48.60 ± 3.56	89.96 ± 4.02	s	s	H	H	s	s	s	s	s
DJY1×NIL*S20*	F_1	49.10 ± 4.07	88.40 ± 2.34	s	s	s	s	H	H	s	s	s
DJY1×NIL*S37*(t)	F_1	58.91 ± 8.27	44.12 ± 1.32	s	s	s	s	s	s	H	H	s
DJY1×NIL*S38*(t)	F_1	46.79 ± 1.51	82.65 ± 5.11	s	s	s	s	s	s	s	s	H
DJY1×NIL*S39*(t)	F_1	47.32 ± 2.82	75.72 ± 12.71	s	s	s	s	s	s	s	s	s
DJY1×NIL*S1S20*	F_1	33.81 ± 1.71	42.69 ± 9.30	H	H	s	s	H	H	s	s	s
DJY1×NIL*S1S37*(t)	F_1	31.46 ± 10.0	50.19 ± 4.74	H	H	s	s	s	s	H	H	s
DJY1×NIL*S1S20S37*(t)	F_1	3.35(n = 1)	0.00	H	H	s	s	H	H	H	H	s

[a] Locus name and chromosome

[b] s, H and g indicated DJY1-homozygous, heterozygous and *O. glaberrima*-homozygous genotypes, respectively.

Hybrid sterile effect caused by S loci

DJY1, the recurrent parent and tested line of *O. sativa*, was crossed with its nine NILs, NIL*S1*, NIL*S19*, NIL*S20*, NIL*S37*(t), NIL*S38*(t), NIL*S39*(t), NIL*S1S20*, NIL*S1S37*(t) and NIL*S1S20S37*(t). As each NIL had the same DJY1 background and the difference was only on the specific chromosome fragment harboring the specific sterile *S* allele, the effect of genetic background could be readily eliminated and the difference between DJY1 and the NILs originated from the *S* locus. For the NILs with the single hybrid sterile locus, the pollen and spikelets fertility of F_1 plants were semi-sterile in the crosses between DJY1 and the NIL*S1*, NIL*S37*(t); the pollen grains were semi-sterile and the spikelet fertility were normal in the crosses between DJY1 and the NIL*S19*, NIL*S20*, NIL*S38*(t) and NIL*S39*(t) (Table 1). These results indicated that the *S1* and *S37*(t) acted as the "gamete eliminator" and *S19*, *S20*, *S38*(t) and *S39*(t) acted as the "pollen killer" which is correspond with previous report. The genetic pattern of those sterility loci followed one-locus sporophyte-gametophyte interaction model[13].

When the NILs with multiple *S* alleles were crossed with DJY1, the pollen fertility was significantly lower than that of the single *S* allele. The NIL*S1S20* and NIL*S1S37*(t) containing two hybrid sterile genes caused about two-thirds of pollen abortion in hybrids and the F_1 pollen grain fertility were 33.81% and 31.46%, respectively. In particular, the pollen grain of F_1 plants was 3.35% in the cross between DJY1 and the NIL*S1S20S37*(t) (Table 1), which was the lowest among the hybrid cross combinations. These results showed that the cumulative effect of these sterile loci in F_1 hybrids was remarkable. When NIL*S1* and NIL*S37*(t) crossed with DJY1, the pollen and spikelet fertility of F_1 plant were semi-fertility (Table 1) which followed the gamete eliminate model. However, the spikelet fertility of F_1 plants was still semi-fertility when the NIL*S1S37*(t) crossed with DJY1 (Table 1). It is unclear how these two loci are involved in interaction.

Bridge effect of the S loci

In addition, DJY1 and its nine NILs were used as female and male parents to make reciprocal crosses with two *O. glaberrima* accessions in order to evaluate the bridge effect of the *S* loci, and the crosses of *O. glaberrima* with DJY1 were used as the control. The F_1 plants were backcrossed as females to their corresponding NILs untill the BC_2F_1 generation was achieved.

Interspecific hybrids between the NILs and two *O. glaberrima* accessions were obtained and hybrids between DJY1 and *O. glaberrima* accessions were used as the control. The average pollen fertility of each cross was evaluated in the F_1 generation.

There was no significant difference in pollen fertility between reciprocal F_1s of all combinations (P = 0.94), indicating that no cytoplasmic effect was involved in hybrid sterility between the two Africa cultivated rice accessions and the Asian cultivated rice variety DJY1 (data not shown). The difference of F_1 pollen fertility between the two interspecific test crosses, DJY1 1×IRGC102263 and DJY1×IRGC103469 was not significant (P = 0.08), indicating that the compatibility of DJY1 with the two African cultivated rice varieties was consistent. The F_1 pollen fertility data of reciprocal crosses between each NIL (or recurent parent) and two Afrian rice accessions was merged to obtain larger sample size.

The F_1 progenies between the six NILs carrying the single *O. glaberrima* hybrid sterile alleles and the *O. glaberrima* accessions showed significantly different on pollen fertility (Table 2). Student's t test indicated that when NIL*S19*, NIL*S20*, and NIL*S37*(t) were crossed with the *O. glaberrima* accessions, the average pollen fertility of F_1 was 0.49%, 1.72%, 0.85%, respectively, which was notably higher than that of the control (0.15%). Among them, *S20* locus had the largest effect on improving pollen fertility of interspecific F_1 hybrids, followed by *S37*(t), and *S19* is only significant at 0.05 level. While the progenies of other NILs, NIL*S1*, NIL*S38*(t) and NIL*S39*(t), were similar to the control.

Table 2
Pollen fertility of F1 plants from the cross between two O. glaberrima accessions and NILs with single hybrid sterile genes.

Crosses	Mean ± SD (%)	Least value (%)	Maximum value (%)	Plant number
DJY1x*O. glaberrima*	0.15 ± 0.39	0.00	1.31	19
NIL*S1*x*O. glaberrima*	0.12 ± 0.25	0.00	0.72	13
NIL*S19*x*O. glaberrima*	0.49 ± 0.86*	0.00	2.05	7
NIL*S20*x*O. glaberrima*	1.72 ± 2.39**	0.00	8.42	20
NIL*S37*(t)x*O. glaberrima*	0.85 ± 0.94**	0.00	4.10	23
NIL*S38*(t)x*O. glaberrima*	0.00 ± 0.00	0.00	0.00	5
NIL*S39*(t)x*O. glaberrima*	0.10 ± 0.24	0.00	0.60	6
* means significant at 0.05 level; ** means significant at 0.01 level				

The pollen fertility of F_1 hybrids between the NILs with multiple sterile loci and the African rice accessions were notably higher than that of control (the crosses between recurrent parent and the African accessions). The F_1 pollen fertility was 5.96% in the hybrids between NIL*S1S37*(t) and *O. glaberrima* accessions; and 4.73% in the hybrids between NIL*S1S20* and *O. glaberrima* accessions. The tri-gene polymerization NIL*S1S20S37*(t) produced notably higher pollen fertility (3.90%) of F_1 hybrids than that of NILs with single locus. However, the F_1 pollen fertility of NIL*S1S20S37*(t) showed no significant difference from those of the F_1 progenies of two gene NIL*S1S20* and NIL*S1S37*(t) (Table 3).

Table 3
Pollen fertility of F1 hybrids between two O. glaberrima accessions and NILs with multiple hybrid sterile genes

Crosses	Mean ± SD(%)	Least value(%)	Maximum value(%)	Plant number
DJY1x*O. glaberrima*	0.15 ± 0.39	0.00	1.31	19
NIL*S1S20*x*O. glaberrima*	4.73 ± 3.11**	1.67	8.85	5
NIL*S1S37*(t)x*O. glaberrima*	5.96 ± 0.70**	5.46	6.45	2
NIL*S1S20S37*(t)x*O. glaberrima*	3.90 ± 2.10**	1.83	7.35	7
* means significant at 0.05 level; ** means significant at 0.01 level				

The results indicated that the multiple hybrid sterile alleles from *O. glaberrima* gave better bridging effective than those of single allele.

Backcrossing can be used to improve pollen fertility of interspecific hybrids after the hybridization

For the NILs with single hybrid sterile locus from *O. glaberrima*, the highest pollen fertility reached 85.81% in the BC_1F_1 population from the cross of NIL*S1* and *O. glaberrima*, which was more than three times of the control (Table 4). Compared with the F_1 generation, the average pollen fertility of BC_1F_1 generation increased significantly. The average pollen fertility of the BC_1F_1 individuals involving six NILs carrying the single sterile allele ranged from 8.14–13.77% and the average pollen fertility of the control's is 5.06%. However, the average pollen fertility of the six NILs carrying the single sterile allele did not notably differ from the control. It is because of the large standard deviation value in the BC_1F_1 segregation population. The average pollen fertility in BC_1F_1 generations showed that the plants harboring the multiple sterile alleles from *O. glaberrima* were notably more fertile than that of lacking them. When *O. glaberrima* accessions were crossed with NIL*S1S20* and NIL*S1S37*(t) which harboring two sterile alleles from *O. glaberrima*, their average pollen fertility in the BC_1F_1 population was 44.04% and 49.50%, respectively; and the pollen fertility was range from 3.11–97.00% and 19.57–81.71%, respectively (Table 4). The result indicated that the pyramided lines with multiple hybrid sterility alleles still had the more prominent bridge effect in the BC_1F_1 generation.

Table 4
Pollen fertility of BC1F1 from the crosses between NILs and two O. glaberrima accessions

Crosses	Average(%)	Least value(%)	Maximum value(%)	Plant number
DJY1x*O. glaberrima*	5.06 ± 4.89	0.00	25.85	69
NIL*S1*x*O. glaberrima*	11.87 ± 15.65	0.00	85.81	207
NIL*S19*x*O. glaberrima*	9.87 ± 11.19	0.00	42.96	43
NIL*S20*x*O. glaberrima*	10.27 ± 11.41	0.00	55.08	98
NIL*S37*(t)x*O. glaberrima*	8.14 ± 10.21	0.00	46.34	47
NIL*S38*(t)x*O. glaberrima*	13.77 ± 18.27	0.00	53.69	14
NIL*S39*(t)x*O. glaberrima*	8.36 ± 10.99	0.00	36.09	24
NIL*S1S20*x*O. glaberrima*	44.04 ± 26.60**	3.11	97.00	50
NIL*S1S37*(t)x*O. glaberrima*	49.50 ± 25.90**	19.57	81.71	4
* means significant at 0.05 level; ** means significant at 0.01 level				

The average pollen fertility was notably higher in the BC_2F_1 generation than those of in the BC_1F_1 generation. In the crosses between NIL*S1* and the *O. glaberrima* accessions, the average pollen fertility (51.01%) is notable higher than that of the control. The pyramiding lines with two hybrid sterile alleles still showed good performance in the BC_2F_1 generation. The hybrids between NIL*S1S20* and *O. glaberrima* accessions had the highest average pollen grain fertility (78.92%). It is worth mentioning that the maximum pollen fertility reached normal range (> 95%) in all the hybridization crosses in BC_2F_1 generation (Table 5).

Table 5
Pollen fertility of BC2F1 from the crosses between NILs and two O. glaberrima accessions

NILs	Average (%)	Standard deviation	Least value (%)	Maximum value (%)	Plant number
DJY1x*O. glaberrima*	30.93	21.89	0.00	99.29	516
NIL*S1*x*O. glaberrima*	51.01**	34.38	0.00	99.40	1018
NIL*S19*x*O. glaberrima*	37.47	23.45	0.00	99.18	65
NIL*S20*x*O. glaberrima*	39.53	26.48	0.00	99.40	340
NIL*S37*(t)x*O. glaberrima*	31.83	20.69	0.00	98.91	248
NIL*S38*(t)x*O. glaberrima*	26.71	27.43	0.00	99.39	152
NIL*S39*(t)x*O. glaberrima*	31.23	20.53	0.00	98.51	163
NIL*S1S20*x*O. glaberrima*	78.92**	26.92	0.00	99.41	663
* means significant at 0.05 level; ** means significant at 0.01 level					

Discussion

Hybrid sterility is the biggest obstacle in the rice hybridization breeding project. Hybrid sterility is controlled by multiple hybrid sterile nuclear genes, most of these genes conform to the inheritance of a single Mendelian factor, and some hybrid sterile genes have epistatic interaction. So far, more than 50 hybrid sterile loci were reported, among which more than 10 hybrid sterile loci were reported between the two cultivated species, *O. sativa* and *O. glaberrima*[5]. Our study showed that the presence of a single hybrid sterile gene can cause about 50% of gametes sterility, while the presence of two hybrid sterile genes can cause about two-thirds of male gametes sterility. If three hybrid sterile genes are involved, the pollen fertility of that hybrid F_1 will be less than 5%. Up to now, it is known that interspecific hybrid sterility of cultivated rice involves at least 10 genes. When Asian cultivated rice is crossed with African cultivated rice, its F_1 generation has almost no fertile pollen. Therefore, interspecific hybrid sterility has always been the focus and difficulty in the rice genetic improvement.

The hybrid sterility also occurs frequently in the inter-subspecific hybridization crosses in rice and the hybrid sterility is mainly affected by five loci involving four for F_1 male sterility and one for F_1 female sterility[14]. The *indica*-compatible *japonica* lines (ICJLs) were developed by pyramiding four *indica* allele and one neutral allele in *japonica* genetic background through marker-assisted selection. When the *indica*-compatible *japonica* lines were test-crossed with a set of typical *indica* and *japonica* varieties, the results indicated that the ICJLs were compatible with *indica* while incompatible with *japonica* rice. The study showed a great promise of overcoming the intersubspecific hybrid sterility by developing pyramiding lines at hybrid sterility loci[14].

Based on known genetic information, if the sterile loci in a given *O. sativa* background could be substituted by the neutral alleles or the corresponding alleles from *O. glaberrima*, it would be possible to overcome the interspecific hybrid sterility between *O. sativa* and *O. glaberrima*[15, 16]. Deng et al. (2010) tested the feasibility of developing a “bridge parent” carrying the *S1-glab* allele from *O. glaberrima* in an *O. sativa* background[9]. The *O. sativa* lines carrying the *S1-glab* allele from *O. glaberrima* produced F_1 progeny with notably higher pollen grain fertility(3.52–5.37%) and egg fertility 9.33–10.54% than their controls (1.88% and 6.31%). This suggested that the *O. sativa* lines carrying the *S1-glab* allele from *O. glaberrima* could be used to improve the fertility of hybrids between *O. glaberrima* and *O. sativa* while the bridge effect of other loci remained unknown. In our case, it was confirmed that some of the “bridge parents” carrying a single sterile allele from *O. glaberrima* produced interspecific F_1 progenies with improved pollen fertility. Furthermore, present study showed that the pyramiding lines of multiple hybrid sterile alleles could significantly improve the F_1 pollen fertility than those with single hybrid sterility locus. In the presence of two alleles of *O. glaberrima*, the pollen fertility of hybrid F_1 increased 5–10 times compared with that of single gene. In fact, in the interspecific hybrid breeding project, increasing pollen fertility from 0 to 5% has no remarkable breeding value. Because the interspecific hybridization involves more hybrid sterile genes than that of the inter-subspecific[15], the “bridge effect” of a single hybrid sterile allele is very limited, even the pyramiding lines with two or three hybrid sterile alleles cannot obtain ideal "bridge effect".

In the test crosses of the *indica*-compatible *japonica* lines with *indica*, the result showed that the F_1 pollen and spikelet fertility reversed close to complete fertility when the *indica*-compatible *japonica* lines pyramided with four loci for “pollen killer” and one for “embryo sac killer”[14]. It can be deduced that the pyramiding lines with *O. glaberrima* alleles on five loci is still not enough on reversing the pollen and spikelet fertility to normal. It is worth further study from the perspective of practical application that how many hybrid sterile loci need to be pyramided to achieve the ideal bridging effect.

Backcrossing is a methods used to improve homogeneity of genetic background, and minimize the genetic variation inducing by genetic background. In our case, pollen fertility in BC_1F_1 generations showed that the plants harboring the sterile allele from *O. glaberrima* were notably more fertile than plants lacking the sterile allele from *O. glaberrima* when backcrossed to the DJY1. Furthermore, the average pollen fertility of the six NILs carrying a single fertile allele did not notably differ from each other, which indicated that all single alleles had near same effect on improving fertility of interspecific hybrids in BC_1F_1. Conversely, similar to most phenotypic variations, interspecific hybrid sterility was shown to be a quantitative trait controlled by multiple genes. Although the bridge lines with the single *O. glaberrima* allele were shown to produce interspecific hybrids with improved fertility, other sterile loci were still heterozygous, which reduced fertility in all the F_1 populations. It has been shown that the introgression at a single sterile locus is not enough to overcome F_1 sterility via elimination of single sterile locus action, which is similar to previous reports[9, 17]. Backcrossing can increase pollen and spikelet fertility of the progeny, while the valuable genes linkage with the sterile loci contained in *O. glaberrima* will naturally disappear along with hybrid sterility in the first cross. All three NILs with multiple *O.*

glaberrima alleles produced interspecific F_1 progeny with notably higher pollen grain fertility than the control. This indicates that the strategy of developing *O. sativa* lines possessing as multiple sterile alleles from *O. glaberrima* as possible to overcome the hybrid sterility between these two species was effective. However, the NIL*S1S20S37*(t) produced interspecific F_1 progeny with notable lower pollen grain fertility than the F_1 progeny of NIL*S1S20* and NIL*S1S37*(t). The epistatic interaction between different loci may involved in the interspecific hybrid. Several epistatic interactions between different loci causing hybrid sterility and hybrid breakdown were reported[10, 18, 19, 20, 21]. More efforts are needed to elucidate the effect of various combinations of multiple pyramiding hybrid sterile bridge parents, and to dissect their interaction or epistatic effect among hybrid sterility loci.

Materials and Methods

Plant materials and growth conditions

An *O. sativa japonica* subsp. cultivar Dianjingyou 1 (DJY1) from Yunnan Academy of Agricultural Sciences (YAAS) was used as the recurrent parent. Two *O. glaberrima* accessions, IRGC102263 and IRGC103469, from International rice Research Institute (IRRI) were used as the tested lines of *O. glaberrima* in this study.

All materials were planted at the Winter Breeding Station, YAAS, Sanya, Hainan Province, P. R. China. The first cropping season was from November to April of the following year, and the second cropping season was from July to October.

Phenotypic evaluation

Pollen fertility for all parental lines, F_1, BC_1F_1 and BC_2F_1 plants was investigated following the instructions of Zhu[22]. Pollen grain fertility was measured using anthers collected from spikelets at 1 to 2 days before anthesis and stored in 70% ethanol[23]. Three to four anthers per floret per plant were mixed and stained with 1% I-KI solution, and more than 300 pollen grains were observed under a light microscope. Sterile types were further classified as typical, spherical or stained abortion types[24]. Three independent microscopic fields were scored for estimation of the percentage of the four types of pollen grains in each plant. Spikelet fertility was scored as the fertilized spikelet rate of three to five panicles on each plant.

Molecular marker and assay

The SSR markers used in this study for developing the NILs carrying single and mutiple alleles of *O. glaberrima* were selected on rice microsatellite maps[25]. A whole-genome SNP array(6k) of rice designed by Cornell University was used to survey the genome of the NILs.

Statistical analysis

Statistical analysis of the data was performed using one-way ANOVA, and the Student's test was used for further pairwise comparisons if ANOVA differences were significant. Pollen and spikelet fertility data as a percentage was transformed by function arcsine square root before the analysis but are listed as percentages.

Statements and Declarations

Data availability statement: All relevant data are within the paper.

Acknowledgments: We would like to thank Dr. Huihui Yu for his help in the process of making chromosome maps based on R Language. This research was funded by National Natural Science Foundation of China, grant number C130501, 31860372, 32160489, and 31000704; Basic Research Foundation of Yunnan Provincial Science and Technology Department, China, grant number 202201AS070072, 202101AS070286; Technology Talent and Platform program of Yunnan Provincial Science and Technology Department, China, grant number 202205AC160057; Applied Basic Research Foundation of Yunnan Academy of Agricultural Sciences JZ201801; and the Yunnan Seed Industrialization Laboratory Program. We would like to thank Dr. Huihui Yu for his help in the process of making chromosome maps based on R Language.

Author Contributions: Conceptualization, D.T.and J. L.; experiment implementation, J.L. and J.Z.; investigation, J.L., P.X., X.D., W.D., Y.Y, Y.Z., Y.L., Q.P.; writing—original draft preparation, J.L.; writing—revise and editing, D.T. and J.Z.. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The author(s) declare no competing interest.

Ethics declarations: The plant collection and use was in accordance with all the relevant guidelines.

Permissions statement: The rice cultivars involved in this paper have permission.

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Figures

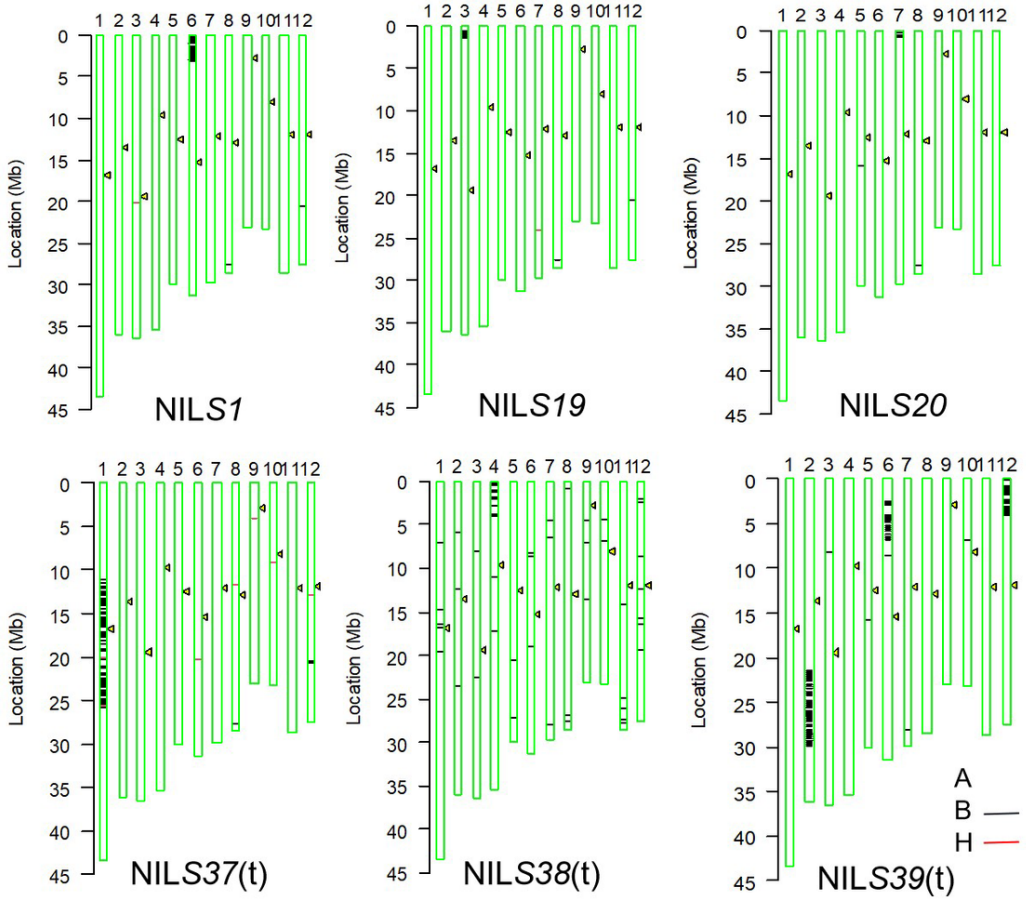

Figure 1. The chromosome introgression map of six NILs, black represented homozygous for *O. glaberrima* alleles; red represented heterozygous and white represented homozygous for DJY1 alleles.

Figure 1

The chromosome introgression map of six NILs, black represented homozygous for *O. glaberrima* alleles; red represented heterozygous and white represented homozygous for DJY1 alleles.